N-terminal α Dystroglycan (αDG-N): A Potential Serum Biomarker for Duchenne Muscular Dystrophy

Kelly E. Crowe[#], Guohong Shao[^], Kevin M. Flanigan[^+] and Paul T. Martin[^+*]

*Author for Correspondence

^Center for Gene Therapy

The Research Institute at Nationwide Children's Hospital

+Department of Pediatrics

The Ohio State University College of Medicine

700 Children's Drive

Columbus, OH 43205

Paul.Martin@nationwidechildrens.org

#Graduate Program in Molecular Cellular and Developmental Biology

The Ohio State University

The final publication is available at IOS Press through http://dx.doi.org/10.3233/JND-150127

Abstract

Background: Duchenne Muscular Dystrophy (DMD) is a severe, progressive, neuromuscular disorder of childhood. While a number of serum factors have been identified as potential biomarkers of DMD, none, as yet, are proteins within the dystrophin-associated glycoprotein (DAG) complex. **Objectives:** We have developed an immobilized serum ELISA assay to measure the expression of a constitutively cleaved and secreted component of the DAG complex, the N-terminal domain of α dystroglycan (αDG-N), and assayed relative expression in serum from muscular dystrophy patients and normal controls. **Methods:** ELISAs of immobilized patient or mouse serum and Western blots were used to assess αDG-N expression. **Results:** Immobilization of diluted serum on ELISA plates was important for this assay, as methods to measure serum αDG-N in solution were less robust. αDG-N ELISA signals were significantly reduced in DMD serum (27±3% decrease, n=9, p<0.001) relative to serum from otherwise normal controls (n=38), and calculated serum αDG-N DMD concentrations were reduced in DMD relative to normal (p<0.01) and Becker Muscular Dystrophy (n=11, p<0.05) patient serum. By contrast, ELISA signals from patients with Inclusion Body Myositis were not different than normal (4±3% decrease, n=8, p=0.99). αDG-N serum signals were also significantly reduced in utrophin-deficient mdx mice as compared to mdx and wild type mice. **Conclusions:** Our results are the first demonstration of a component of the DAG complex as a potential serum biomarker in DMD. Such a serum measure could be further developed as a tool to help reflect overall muscle DAG complex expression or stability.

Key words: dystroglycan, muscular dystrophy, biomarker, dystrophin, utrophin

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, progressive, X-linked muscle disease of childhood caused by mutations in the *DMD* gene that lead to loss of expression of the dystrophin protein(1, 2). Diagnosis of DMD typically occurs shortly after children begin to ambulate, with loss of ambulation occurring in the early teenage years and death typically ensuing in the third decade of life due to respiratory insufficiency and/or cardiomyopathy(3). Becker Muscular Dystrophy (BMD) is also caused by mutations in the *DMD* gene, but muscle cells in BMD patients are able to express mutated partially functional dystrophin protein that allows for a generally less severe clinical phenotype(4). Loss of dystrophin expression increases the frailty of muscle membranes, thereby leading to membrane rupture and loss of calcium homeostasis that ultimately causes muscle wasting(5-8). Such membrane frailty in DMD results, in part, from the failure of dystrophin protein to properly anchor components of the dystrophin-associated glycoprotein (DAG) complex in the sarcolemmal membrane, which in turn weakens connections between the extracellular matrix, the muscle membrane, and the intracellular F-actin cytoskeleton(9-13).

The classic serum biomarker for DMD is increased enzyme activity of creatine kinase (CK), a muscle protein that is released into the serum as the result of membrane perforation(14). Elevation of serum CK activity is evident in DMD patients even at birth, and serum CK levels are typically ten to one hundred-fold higher than those found in normal subjects(15, 16). Serum CK activity, however, can also be elevated by exercise in non-dystrophic subjects and by a variety of other muscle insults, such as viral infections. As such, elevated serum CK activity is not a specific marker for DMD and elevations can be highly variable even within individual DMD subjects (17, 18). Similarly, increased levels of serum cardiac troponin can be found in

DMD patients, who develop dilated cardiomyopathy, but this again can be found in a variety of other cardiac events not specific to DMD(19).

Because of the need to identify markers of global DMD disease that would not require a muscle biopsy, a number of proteomics studies have been initiated to identify further serum markers that change in DMD. While certainly not an exhaustive list, additional protein markers identified as elevated in DMD serum include fibronectin(20), titin(21), myomesin 3(21), filamin C(21), actin(21), phosphoglycerate mutase 2(21), myoglobin(21, 22), fibrinogen(21, 22), matrix metalloproteinase 9(23), tissue inhibitor of matrix metalloproteinase 1(23), osteopontin(24) and follistatin(25). In addition, some urine proteins, including titin(26), and several serum micro-RNAs, including miR-1(27, 28), miR-133(27, 28) and miR-206(27, 28), are elevated in DMD. This list suggests that a myriad of molecular changes can arise as muscle damage occurs in DMD patients. Some of these markers are further suggested to have altered expression as muscle pathology and clinical findings progress(17). What is notable about this list, as yet, is that none of the proteins are known to directly interact with dystrophin within the DAG complex, which is why we have undertaken the current study to assay a component of the DAG complex in DMD patient serum.

α dystroglycan can be cleaved in muscle cells to generate a secreted N-terminal fragment, αDG-N, that Matsumura and colleagues first showed can be expressed in human serum as a glycosylated protein(29). αDG-N is normally cleaved by furin in the Golgi apparatus as dystroglycan is being secreted to the cell surface, and αDG-N is removed from α dystroglycan in skeletal muscle(12, 30, 31). Thus, αDG-N is released by muscle, but is also likely released by non-muscle cells where dystroglycan is also normally expressed. Because dystroglycan protein is expressed in many tissues, serum αDG-N likely reflects a collection of cleaved dystroglycan

proteins emanating from various tissues throughout the body, however, skeletal muscle comprises a significant fraction of this tissue. In addition to serum, αDG-N has also been identified in the cerebrospinal fluid, lachrymal fluid and urine fluid(32, 33). αDG-N expression in the cerebrospinal fluid is elevated in patients with Lyme neuroborrelosis, suggesting that αDG-N expression may be altered in certain disease states(32), an idea proposed by Brancaccio and colleagues for the muscular dystrophies(34). Here we have developed a serum ELISA to assess the relative expression of serum αDG-N in patients with DMD relative to patients with BMD, IBM, which is a myopathy where DAG complex expression is not typically altered, or relative to otherwise normal controls. We find that DMD patients have reduced serum αDG-N expression suggestive of altered stability or expression of the DAG complex in DMD that might be exploited with future development to aid in DMD diagnosis or in the assessment of certain DMD therapies.

RESULTS

Development of an αDG-N ELISA assay

We compared three approaches to assaying serum αDG-N expression using an ELISA assay (Fig. 1). In the first approach, we immobilized serum at high dilutions or immobilized purified αDG-N directly onto the ELISA plate and then probed amounts of αDG-N using 2A3, a mouse monoclonal antibody specific to this region of the protein(29). 2A3 binding was then indirectly visualized by binding of an anti-mouse IgG2a coupled to horseradish peroxidase (HRP), followed by a standard HRP color enzyme reaction and reading of absorbance at 450nm (OD450) in an ELISA plate reader. In the second and third assays, we tried an indirect competition ELISA assay and a sandwich ELISA assay to measure αDG-N levels in serum that was added to an ELISA plate immobilized with 2A3. For the indirect ELISA, we combined a constant amount of biotinylated αDG-N with differing amounts of unlabeled αDG-N or serum and assessed loss of signal resulting from increased competitive binding of unlabeled αDG-N. After washing, streptavidin-HRP was added to develop the signal using standard color development for HRP enzyme activity. For the sandwich method, we again first immobilized 2A3 on the ELISA plate. Purified αDG-N or serum was then added, washed, and αDG-N binding visualized by addition of biotinylated 3B4, a second αDG-N antibody, followed by streptavidin-HRP and development as before. This sandwich assay is almost identical to that used in a study recently published assay by Nie and colleagues to measure αDG-N in human uterine fluid(35).

In Fig. 1, we show examples of standard curves for each type of assay using purified, recombinant αDG-N protein. When αDG-N was immobilized on the ELISA plate in different

amounts, we found that 2A3 binding could be correlated with different amounts of immobilized αDG-N(Fig. 1A). For this serum-immobilized assay, the upper limit of quantification (ULOQ) was 5ng and the lower limit of quantification (LLOQ) was 0.16ng. Similarly, for the indirect ELISA, we could show a correlation between loss of signal using increasing amounts of non-biotinylated αDG-N in a range from 2ng to 15ng (Fig. 1B). Surprisingly, we found no correlation in antibody binding from the sandwich ELISA assay using recombinant αDG-N (Fig. 1C). We did, however, find a correlation when a partial αDG-N protein fragment linked to glutathione-S-transferase (αDG-GST) was used (Fig. 1C). This was the protein previously described by Nie and colleagues in their αDG-N sandwich ELISA assay(35). αDG-GST has only amino acids 31-141 of the expected αDG-N sequence, which begins at amino acid 30 after the signal peptide and ends at amino acid 312(31). In addition, this shorter αDG-GST protein was made in *E. coli* and so would not be glycosylated. By contrast, the recombinant αDG-N protein we had made in transfected HEK293 cells corresponded to the entire amino acid 30-312 protein sequence expected for the furin-cleaved αDG-N fragment and was glycosylated, as has been previously reported by Matsumura and colleagues(29). We found we could generate a standard curve using the ELISA assay with 2A3 (Fig. 1D) or biotinylated 3B4 (Fig. 1E) antibody using recombinant, full-length αDG-N, but in both instances, pre-incubation with the other antibody in solution eliminated all such binding (Fig. 1D and E). This suggests that the binding site for these antibodies on native αDG-N are incompatible with use in a sandwich ELISA, as both antibodies cannot both simultaneously recognize native αDG-N. As no other αDG-N antibodies were available, we did not pursue the sandwich method further.

We next compared spike-ins of known amounts of purified αDG-N protein, adding 0.5, 1.0 or 1.5ng (for serum-immobilized assay) or 1.25 or 2.5ng of αDG-N (for competition assay)

from 2 normal human and 2 DMD patient sera samples to determine recovery precision of added αDG-N. For the serum-immobilized assay, we measured a 56±4% recovery yield of αDG-N from normal human serum and a 55±7% recovery yield of αDG-N from DMD serum, and this yield was roughly equivalent at all added αDG-N amounts. In addition, these yields were not significantly different between normal human and DMD serum. For the competition ELISA assay, we measured a cumulative recovery yield of 189±37% for αDG-N from normal human serum and a recovery yield of 88±11% for αDG-N from DMD serum. These yields were in fact significantly different (p=0.03). In other experiments, the degree of yield changes between DMD and normal was sometimes even more pronounced if serum amounts added reached the lower OD450 signal levels on the standard curve (not shown). Because the yield of spiked signal was beyond the expected signal for normal serum for the competition ELISA, and because DMD and normal sera also showed significantly different responses, we did not pursue this assay further. We therefore proceeded to investigate differential αDG-N levels using the serum immobilization assay.

αDG-N is decreased in the serum of patients with DMD

We performed serum-immobilized ELISAs to measure αDG-N levels in human serum from patients with Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), otherwise normal patient controls, and a myopathy unrelated to the DAG complex, Inclusion Body Myositis (IBM) (Fig 2). A summary of relevant patient information is included in Supplemental Table 1. This includes the fact that 8 of 9 DMD patients, 2 of 11 BMD patients, and 0 of 8 IBM patients had been treated with corticosteroids in the 3 months prior to the taking

of the serum sample. There were no significant changes in comparing any disease group with regard to corticosteroid use (not shown). Serum dilutions sometimes had to be altered to maintain all signals within the linear range of the standard curve, but were generally done at or near a 1:80,000 per plate, which was the predominant dilution used for human samples. Assuming a serum concentration of 80mg/mL protein, a 1:80,000 serum dilution would result in 100ng of serum protein being immobilized per well of a 96-well ELISA plate. We ascribe the need to change serum dilution for some experiments to the quality of immobilization on various lots of ELISA plates and to the relatively tight range of the αDG-N standard curve. In each experiment, however, all normal and patient serum samples were identically diluted. We analyzed the data in two different ways. First, we analyzed the absolute relative change in OD450 signal between serum samples (Fig. 2A). To do this, we reported the fold-changes in each signal relative to the average signal for all otherwise normal samples in each experiment, set to a value of 1. 3-7 replicate experiments were done per sample, each with duplicate measures per assay. We found that patients with DMD showed a significant reduction (27±3% decrease from normal, n=9) in OD450 signal for αDG-N compared to otherwise normal patients ($p \leq 0.001$, n=38). BMD patients showed an intermediate level of reduction αDG-N in the serum (14±2% decrease from normal, n=11), statistically differing from both otherwise normal patients ($p \leq 0.01$) and DMD patients ($p \leq 0.05$). In contrast, IBM patients, who show adult-onset progressive muscle wasting without major DAG complex alterations(36), showed an insignificant change from normal patients (4±3% decrease from normal, p= 0.99, n=8). Because DMD patients were younger than BMD patients (average age 10 versus 19, respectively), and because IBM patients were significantly older than both of these groups (average age 57), we performed linear regression adjusting for age and gender when comparing all disease groups to

determine significance. In analyzing the variability of individual measures, we found that the serum-immobilized assay showed relatively robust reproducibility. Considering all samples, the overall intra-assay coefficient of variation (CV) was 3.8% and the inter-assay CV was 16.3%.

We next compared measures of αDG-N concentrations derived from the standard curves done with each serum-immobilized assay (Fig. 2B). Because we had to occasionally use a different standard dilution for all of the samples to fit the OD450 signals within the range of the standard curve, we expected these values, which must take into account differing inter-assay dilution factors, to be more varied. While a few measures were more variable due to changed dilution factor, the serum αDG-N concentration was still significantly reduced in DMD serum compared to normal serum as well as to BMD serum (Fig. 2B), with the overall level of reduction of αDG-N expression ranging from 65-70% for all such comparisons. For calculations of αDG-N serum concentrations, the intra-assay CV remained low (4.5%), but because of the need to use different dilution factors for certain experiments to maintain linearity of all samples on the standard curve, the inter-assay CV was poor (73%).

We also validated changed expression of αDG-N in DMD via Western blot (Fig. 3). While more qualitative than an ELISA, levels of αDG-N were decreased in serum from patients with DMD as compared to age-matched otherwise normal male patients (Fig. 3A). Immunoblots for fetuin, an abundant serum protein whose expression should not be altered in DMD, were used as a control for serum protein loading and transfer. The 2A3 antibody recognized several protein bands in human serum migrating between the 39kDa and 51kDa molecular weight markers. Because loading of even 1□L of serum, as was done here, leads to warping of protein bands due to the large amounts of protein loaded, we could not discern the exact molecular weights of these species; however, these species did migrate in the same range as was found for 2A3

immunoblots of purified recombinant αDG-N purified from secreted HEK293 cells. We found that subjecting purified recombinant αDG-N isolated from secreted HEK293 cells to enzymatic deglycosylation (of both N- and O-linked proteins) reduced the molecular weight of the 2A3-blotted protein from about 45kDa to 39kDa, such that it now equaled the molecular weight recognized by 2A3 in HEK293 cell lysate (Fig. 3B). A similar effect was found when normal and DMD serum were deglycosylated, and serum fetuin also showed reduced molecular weight after this treatment (Fig 3B). 37-45kDa is roughly molecular weight of αDG-N protein species previously published for αDG-N protein in human serum and/or cerebrospinal fluid, with about a 37kDa protein identified after protein deglycosylation, much as was seen here(29, 32, 33).

Serum levels of αDG-N do not differ significantly based on age or gender

Despite the fact that our linear regression analysis of human serum αDG-N ELISAs took age and gender into account, we also plotted relative serum levels of αDG-N versus age for all normal patient samples and found that expression within this group did not significantly change with increasing age (r^2=0.009) (Supplemental Figure 1A). αDG-N serum levels also did not change with increasing age in BMD (r^2=0.147) or DMD (r^2<0.001) samples (Supplemental Figure 1B and 1C, respectively). Additionally, αDG-N serum expression was not changed when otherwise normal patients were grouped by gender (females were 105±3% of males, p=0.2) (Supplemental Figure 1D). These data suggest that serum αDG-N expression is a marker of DMD disease that is independent of age or gender.

Utrn$^{-/-}$mdx mice, but not mdx mice, show decreased expression of serum αDG-N compared to normal mice

We next sought to replicate these findings in the mdx mouse model of DMD (Fig. 4). The mdx mouse, like DMD patients, lacks dystrophin protein expression in muscle cells(37). Surprisingly, we found no significant difference in αDG-N ELISA signal between wild type (WT) and mdx serum (p=0.66, n=10 for mdx and 11 for WT, Fig. 4A). Because mdx mice show far less overall muscle pathology than DMD patients and also have upregulation of utrophin protein (made by the *Utrn* gene), a dystrophin paralog known to compensate for the loss of dystrophin in skeletal muscle by binding and stabilizing the DAG complex(38, 39), we also assayed serum from *Utrn$^{-/-}$*mdx mice. Note that while the mouse and human αDG-N proteins are 92% identical in amino acid sequence, some interspecies differences may exist in comparing the human and mouse measures, as we used αDG-N from the same species, rabbit(13), to generate both sets of standard curves. The rabbit αDG-N protein sequence is 93% identical to the human αDG-N sequence and 91% identical to mouse αDG-N sequence. *Utrn$^{-/-}$*mdx mice generally have far more severe disease pathology than do mdx animals due to the loss of both utrophin and dystrophin protein expression(40, 41). In contrast to mdx mice, we found a robust decrease in serum αDG-N signal in *Utrn$^{-/-}$*mdx mice as compared to wild type or mdx mice (*Utrn$^{-/-}$*mdx signal was reduced by 49±4% compared to wild type, p<0.0001, n=4, Fig. 4A). The cumulative intra-assay CV for these serum measures was 4.0%, while the overall inter-assay CV was 13.1%. These reduced OD450 signals in *Utrn$^{-/-}$*mdx mouse serum correlated with a reduced calculated serum concentration as well, with *Utrn$^{-/-}$*mdx αDG-N concentration reduced by 37±3% compared to wild type, p<0.001) and 31±3% compared to mdx (p<0.01, Fig. 4B). For αDG-N concentration measures, the inter-assay CV was 4.8% and inter-assay CV was 34.4%. These

experiments suggest that utrophin expression may impact αDG-N expression in dystrophin-deficient mouse serum.

DISCUSSION

We have developed an ELISA assay that utilizes immobilized diluted serum to measure levels of a normally cleaved N-terminal fragment of α dystroglycan, αDG-N. In doing so, we have found that αDG-N expression in serum from patients with DMD is significantly reduced relative to serum from otherwise normal patients and to serum from BMD patients. These findings were independent of age, suggesting that αDG-N reduction in DMD is more of a fixed marker of disease than a reflection of some ongoing disease process. There are a number of mechanisms that could give rise to the changed expression of the αDG-N protein fragment in DMD serum (Fig. 5). Reduced serum αDG-N levels may reflect reduced intracellular dystroglycan expression or stability in DMD muscle, reduced αDG-N stability once cleaved in DMD muscle or serum, reduced αDG-N secretion from DMD muscle, or increased αDG-N scavenging in DMD serum. As dystroglycan cleavage to liberate αDG-N in muscle appears to be complete in both normal and DMD muscle(10, 12, 42, 43), it seems unlikely that reduced furin activity would account for changed αDG-N expression. Because αDG-N is immobilized for the ELISA measure done here, reduced αDG-N signals in DMD serum may also reflect increased masking of αDG-N due to increased binding of DMD serum proteins to αDG-N antibody-reactive epitopes.

While it is certainly possible that αDG-N expression in the serum reflects reduced dystrophin expression or reduced dystroglycan protein expression, both of which occur in DMD(1, 2, 43), the fact that the same findings could not be replicated in mdx mice, but were replicated in utrophin-deficient mdx mice, makes such a conclusion problematic. While certainly some differences in serum αDG-N expression could reflect human-mouse muscle differences, there is no doubt that most mdx muscles fail to express dystrophin(1, 37), making it

unlikely that serum αDG-N directly reflects dystrophin expression in skeletal muscle. While this reduction was observed in *Utrn*$^{-/-}$mdx mice, it is also unclear how this might be explained by human-mouse differences. One explanation might be that utrophin is better at stabilizing dystroglycan expression in mouse muscle than it is in human muscle, but there is little evidence to support this possibility.

If changed serum αDG-N expression were to indeed reflect altered dystrophin expression in DMD and BMD patients, then it could be exploited as a global marker of dystrophin protein recovery in therapies aimed at reintroducing a partially functional dystrophin protein to DMD patients. Such therapies include antisense- and morpholino-based exon skipping strategies, such as drisapersen(44, 45) and eteplirsen(46, 47), and also missense read-through therapies such as ataluren(48). All of these types of therapies are plagued by the difficulty that analysis of dystrophin expression in single muscle biopsy does not typically reflect changed dystrophin protein expression in muscles throughout the entire body plan, which is the biomarker needed to best reflect overall drug efficacy(49). While additional work will be required to understand if such a finding can be exploited, it is possible that serum αDG-N, as a marker of muscle dystroglycan stability or expression, may be reduced in DMD patient serum because dystrophin is absent. The fact that BMD and DMD serum αDG-N signals differed from one another also suggests that this may be possible. Unfortunately, the level of decline in αDG-N serum signal in DMD vs. normal, while highly significant, is only 27% of total OD450 signal. While the calculated concentration difference is greater, this is a less robust measure due to the non-linear nature of the standard curves and issues with serum dilution. The lack of a greater overall change in αDG-N signal is likely is the result of the fact that dystroglycan is present in many tissues, for example skin, where dystrophin is not present and where dystroglycan can be

stabilized by other dystrophin-like proteins such as plectin 1(50, 51). Thus, there is likely a dystrophin-independent signal emanating from non-muscle tissues that contributes a significant fraction of serum αDG-N expression. Further work will be required to understand these and other issues that may affect changes in serum αDG-N expression, and whether there may be a unique muscle-specific modification of αDG-N that could be utilized to eliminate non-muscle background signal.

While the data presented here provide for a proof of concept that αDG-N expression is changed in the serum of DMD patients relative to otherwise normal patients, our results will benefit from the analysis of additional cohorts, and the assay we have used would need to be further optimized in order to exploit this measure for large-scale quantitative studies. Because we utilized an ELISA where serum was diluted and directly immobilized on the ELISA plate, we found that the serum dilution factor sometimes had to be altered between experiments in order for all signals to be below the saturation range of the standard curve. Although the serum samples within each plate were always diluted to the same degree, this increased inter-assay CV. Another recent study found that use of a second αDG-N monoclonal antibody, 3B4, in addition to the 2A3 antibody used here, allowed for development of a sandwich ELISA to measure αDG-N levels in solution in human uterine fluid(35). That assay used a non-native and smaller fragment of αDG-N protein to generate a standard curve, and we show here that use of native length and glycosylated αDG-N does not allow for such a sandwich assay using these antibodies; pre-incubation of the native full-length αDG-N with either 2A3 or 3B4 did not allow for recognition of αDG-N by the other antibody. It may be that the shorter protein fragment used previously oligomerized in such a way that more than one identical epitope was available for binding. If so, use of native αDG-N does not appear to allow for this to occur. Were a panel of

monoclonal antibodies to be identified that could be mapped to specific protein domains of αDG-N and shown to recognize non-overlapping protein elements, a sandwich ELISA could be developed, and such an assay might obviate the need for immobilizing serum on the plate. Generation of such antibodies would be very helpful for improving this assay, allowing for a more standardized measurement of αDG-N in the serum.

MATERIALS and METHODS

αDG-N Protein Production and Purification

A cDNA encoding an N-terminal FLAG-tagged α dystroglycan protein, αDG-N (MSALLILALVGAAVADYKDDDDKLAAANSHWPSEPSEAVRDWENQLEASMHSVLSDL HEALPTVVGIPDGTAVVGRSFRVTIPTDLIGSSGEVIKVSTAGKEVLPSWLHWDPQSHTL EGLPLDTDKGVHYISVSAAQLDANGSHIPQTSSVFSIEVYPEDHSEPQSVRAASPDLGEA AASACAAEEPVTVLTVILDADLTKMTPKQRIDLLHRMQSFSEVELHNMKLVPVVNNRL FDMSAFMAGPGNAKKVVENGALLSWKLGCSLNQNSVPDIRGVEAPAREGTMSAQLGY PVVGWHIANKKPPLPKRIR), with the pre-protrypsin signal peptide from the pCMV1-FLAG expression vector, was cloned into the pFLAG-CMV-1 vector using EcoRI and XbaI sites to yield a secreted αDG-N (30-312)(13) amino acid sequence with an N-terminal FLAG tag. The plasmid was transfected into HEK293T cells, and αDG-N was purified from the supernatant via anti-FLAG (M2) affinity chromatography as previously described (52).

Protein Biotinylation

Purified, recombinant αDG-N and 3B4, a monoclonal antibody to αDG-N (Creative Diagnostics; Shirley, NY) were biotinylated using EZ-Link™Sulfo-NHS-Biotin (Thermo Scientific; Waltham, MA). The labeled proteins were subsequently desalted using Zeba™ Desalt Spin Columns (Thermo Scientific) and protein concentrations measured using a modified Bradford assay, as before(53).

Serum αDG-N ELISA Assays

Human serum was obtained from subjects identified within the neuromuscular clinic at Nationwide Children's Hospital under an Institutional Review Board approved protocol (IRB13-00190). Clinical classification of DMD versus BMD was made based upon the concept of "best clinical diagnosis", using an expert clinical diagnosis that combines available information regarding clinical presentation features, family history, and (when available) protein expression. As described elsewhere, this approach takes into account but is not solely based upon mutation class or predicted reading frame(54). Patients who have lost ambulation at younger than age 12 are classified as DMD; those walking at age 15 are classified as BMD, and those who have lost ambulation between ages 12 and 15 are classified as intermediate muscular dystrophy (not included in this study). Mouse studies were conducted under the approved IACUC protocol AR07-00033 at Nationwide Children's Hospital. All mice ranged in age from 3 weeks old to 3 months old. To obtain mouse serum, blood was collection via facial vein and allowed to clot for 1 hour in non-heparinized tubes, then spun at 2,000g for 10 min at 4°C to collect serum.

Before each experiment, a dilution curve was performed with normal, DMD, BMD, and IBM human serum samples, or with wild type, mdx or *Utrn*$^{-/-}$mdx mouse serum samples, to determine at what dilution factor the serum samples would fall within the standard curve. This dilution factor ranged from 1:5,000 to 1:80,000 for different experiments, with most assays with human serum done at 1:80,000 and most assays with mouse serum done at 1:5,000. After the appropriate dilution factor was empirically determined, all serum samples were identically diluted using that dilution factor in 50mM bicarbonate buffer, pH 9.4, and a 100□L volume incubated overnight on 96-well pre-coated microtiter plate (Thermo-Fisher Scientific; Waltham, MA), in some cases with 0.5, 1.0 or 1.5ng of αDG-N added. A standard curve using differing amounts of purified αDG-N, ranging from pgs to 10ng of protein, was also immobilized

overnight on every plate in 100□L of 50mM bicarbonate buffer, pH 9.4. Wells incubated overnight with sodium bicarbonate buffer alone (with no αDG-N) were used as controls for background signal and subtracted from sample values, and these values typically did not exceed 10% of total positive (αDG-N) signal. Plates were blocked with 1% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST), washed, and incubated with 2A3, a monoclonal mouse anti- αDG-N-specific antibody (WH0001605M1; Sigma; St. Louis, MO) at a 1:1000 dilution in blocking buffer. Wells were subsequently washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse Fcγ subclass 2A-specific IgG (Jackson ImmunoResearch; West Grove, PA) at a 1:4000 dilution in blocking buffer. Wells were washed and developed using the Substrate Reagent Pack (R&D Systems; Minneapolis, MN). The reaction was stopped by 2N sulfuric acid after 20 minutes. Plates were measured for absorbance at 450 nm (OD450) on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). After background signal was subtracted for all values, replicates were averaged and compared to the averaged OD450 for normal serum on each plate, yielding a fold-change from normal average for each sample that was independent of signal variability between experiments. In addition, concentrations for all samples were calculated in reference to an immobilized αDG-N standard curve generated for each plate.

For the competition ELISA, 25ng per well of 2A3 was immobilized on 96-well pre-coated microtiter plates overnight in 50 mM sodium bicarbonate buffer, pH 9.4. Subsequently, plates were blocked with 1% BSA in TBST, washed, and incubated with a standard curve of purified αDG-N that was premixed with biotinylated αDG-N at an empirically determined saturating concentration (5ng/well). Serum was diluted 1:100 and added in the presence of 5ng/well biotinylated αDG-N, sometimes with 1.5ng or 2.5ng of non-biotinylated αDG-N added

as a spike-in. Wells were then washed and incubated with streptavidin-HRP (Jackson ImmunoResearch) at a dilution of 1:1000 in blocking buffer, washed, and developed in a manner identical to the serum-immobilized ELISA assay above. Wells coated with 2A3, but where no αDG-N or serum was added, followed by developed as above, were used as controls for background signal and subtracted from sample values. Background signals for the competition ELISA were generally higher than for the immobilized αDG-N ELISA, ranging between 26% and 41% of total positive (αDG-N) signal.

The sandwich ELISA was done in a manner almost identical to that previously described to measure αDG-N in human uterine fluid(35). Here, 50 ng of 2A3 per well was immobilized on 96-well pre-coated microtiter plates overnight in 50 mM sodium bicarbonate buffer, pH 9.4. Subsequently, plates were blocked with 1% BSA in TBST, washed, and incubated with differing amounts of either purified full-length, native, αDG-N, purified as described above, or a partial αDG-N fragment made as a fusion protein with GST in *E. coli* consisting of amino acids 31-141 (αDG-GST, H00001605-Q01-25ug; Novus Biologicals; Littleton, CO). Wells were then washed and incubated with 1ug/ml of a second, biotinylated monoclonal antibody to αDG-N, 3B4 (Creative Diagnostics), washed again and incubated with streptavidin-HRP (Jackson ImmunoResearch) at a dilution of 1:1000 in blocking buffer. After final washes, the assay was developed for HRP activity as described for the serum-immobilized ELISA assay. Wells coated with 2A3 but where no serum or αDG-N was added were developed and background signal subtracted from sample values. Background signal for the sandwich ELISA were very high, sometimes reaching 75% of total positive (αDG-N) signal.

To determine whether 2A3 and 3B4 competed for binding to αDG-N, ELISA plates were coated overnight with 25ng per well of either 2A3 or 3B4 diluted in 50mM sodium

bicarbonate buffer, pH 9.4. Wells were blocked in 1% BSA in TBST. Next, recombinant αDG-N was added in differing amounts ranging from 1pg to 10ng. For the 5ng incubation amount, some samples were first mixed with 1ug/ml of 3B4 (for 2A3-coated plates) or 2A3 (for 3B4-coated plates). Plates were washed and incubated with streptavidin-HRP (Jackson ImmunoResearch) at a dilution of 1:1000 in blocking buffer, washed again and developed as described above.

CV values were determined by the ratio of the standard deviation to the mean for replicates on the same plate (for intra-assay CV) or for the same samples on different plates (for inter-assay CV). Recovery precision values were determined by first subtracting the unspiked result from the spiked result to ascertain the actual spike recovery, which was then compared to the expected spike recovery to determine the recovery yield. Upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) were determined by the highest and lowest values respectively with a curve backfit of 80-120% and an inter-assay CV of <30%.

Western Blots

Total serum proteins from each sample were diluted (by identical amounts) in SDS denaturing buffer and separated on 4-12% gradient SDS-PAGE gels and then transferred to nitrocellulose. 1uL of serum was denatured and run per lane. After transfer, blots were blocked in TBST with 5% non-fat dry milk, then incubated with primary antibody, either anti-αDG-N (2A3; Sigma; St. Louis, MO) or anti-fetuin (orb27630, Biobyt, Cambridge, UK), washed in TBST, incubated with appropriate horseradish peroxidase-conjugated goat anti-mouse IgG_{2A} secondary antibody (Jackson ImmunoResearch, Seattle, WA), washed again, and developed using an ECL developing kit (Amersham, Piscataway, NJ), much as previously described(55).

To remove glycans, recombinant αDG-N purified from transfected HEK293 cell lysate or supernatant, or whole human serum samples, were enzymatically deglycosylated using a protein deglycosylation mix (P6039S, New England Biolabs; Ipswich, MA) to remove both N- and O-linked glycans. Deglycosylated or untreated proteins were then compared by Western blot using 2A3 to probe for αDG-N or an anti-fetuin antibody as above.

Statistics

For analysis of human samples using the serum-immobilized ELISA assay, linear regression with post-hoc Tukey's pairwise comparison was used to assess significance, adjusting for age and gender. For comparison of mouse serum samples using the serum-immobilized ELISA, significance was determined by ANOVA with post-hoc Tukey's pairwise comparison. R square values were determined by linear regression or non-linear regression where appropriate. Statistics were analyzed using GraphPad Prism Version 6.03 (GraphPad Software Inc., La Jolla, CA), save human data, which was analyzed by the Biostatistics Core at Nationwide Children's Hospital.

ACKNOWLEDGEMENTS

We would like to thank Susan Gailey and Krista Kunkler for assistance with obtaining clinical serum samples and Rui Xu for technical assistance with experiments. We would like to thank Han Yin and Igor Dvorchik in the Biostatistics Core at The Research Institute at Nationwide Children's Hospital for their assistance with statistical analysis.

FUNDING

This work was supported by NIH grant R01 AR049722 to PTM.

COMPETING INTERESTS

None of the authors have competing interests to declare.

ABBREVIATION LIST

Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Inclusion Body Myositis (IBM), dystrophin-associated glycoprotein (DAG), enzyme-linked immunosorbent assay (ELISA), creatine kinase (CK), N-terminal α Dystroglycan (αDG-N), wild type (WT), coefficient of variation (CV), upper limit of quantification (ULOQ), lower limit of quantification (LLOQ), absorbance at 450 nm (OD450)

AUTHOR CONTRIBUTIONS

KEC developed and performed the serum ELISA assays and Western blot assays and was involved in writing the manuscript. GS purified αDG-N protein and performed biotinylation experiments. KMF provided human samples and definitive patient diagnoses, and read and revised the manuscript. PTM conceptualized and designed the studies, motivated by the published work of Matsumura and colleagues, and helped in drafting and the editing the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Hoffman EP, Brown RH, Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 1987;51(6):919-28.
2. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell. 1987;50(3):509-17.
3. Manzur AY, Muntoni F. Diagnosis and new treatments in muscular dystrophies. Postgraduate medical journal. 2009;85(1009):622-30.
4. Wein N, Alfano L, Flanigan KM. Genetics and emerging treatments for Duchenne and Becker muscular dystrophy. Pediatric clinics of North America. 2015;62(3):723-42.
5. Brooks SV, Faulkner JA. Contractile properties of skeletal muscles from young, adult and aged mice. J Physiol. 1988;404:71-82.
6. Fong PY, Turner PR, Denetclaw WF, Steinhardt RA. Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. Science. 1990;250(4981):673-6.
7. Turner PR, Westwood T, Regen CM, Steinhardt RA. Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature. 1988;335(6192):735-8.
8. Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, et al. The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. Nature. 1991;352(6335):536-9.
9. Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature. 1990;345(6273):315-9.
10. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol. 1993;122(4):809-23.
11. Martin PT. Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. Glycobiology. 2003;13(8):55R-66R.
12. Ervasti JM, Campbell KP. Membrane organization of the dystrophin-glycoprotein complex. Cell. 1991;66(6):1121-31.
13. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature. 1992;355(6362):696-702.
14. Hyser CL, Griggs RC, Mendell JR, Polakowska R, Quirk S, Brooke MH, et al. Use of serum creatine kinase, pyruvate kinase, and genetic linkage for carrier detection in Duchenne and Becker dystrophy. Neurology. 1987;37(1):4-10.
15. Mendell JR, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, Gastier-Foster J, et al. Evidence-based path to newborn screening for Duchenne muscular dystrophy. Ann Neurol. 2012;71(3):304-13.
16. Ozawa E, Hagiwara Y, Yoshida M. Creatine kinase, cell membrane and Duchenne muscular dystrophy. Mol Cell Biochem. 1999;190(1-2):143-51.
17. Ferlini A, Flanigan KM, Lochmuller H, Muntoni F, t Hoen PA, McNally E. 204th ENMC International Workshop on Biomarkers in Duchenne Muscular Dystrophy 24-26 January 2014, Naarden, The Netherlands. Neuromuscul Disord. 2015;25(2):184-98.

18. Gasper MC, Gilchrist JM. Creatine kinase: a review of its use in the diagnosis of muscle disease. Medicine and health, Rhode Island. 2005;88(11):398, 400-4.
19. McNally EM. New approaches in the therapy of cardiomyopathy in muscular dystrophy. Annu Rev Med. 2007;58:75-88.
20. Cynthia Martin F, Hiller M, Spitali P, Oonk S, Dalebout H, Palmblad M, et al. Fibronectin is a serum biomarker for Duchenne muscular dystrophy. Proteomics Clinical applications. 2014;8(3-4):269-78.
21. Hathout Y, Marathi RL, Rayavarapu S, Zhang A, Brown KJ, Seol H, et al. Discovery of serum protein biomarkers in the mdx mouse model and cross-species comparison to Duchenne muscular dystrophy patients. Hum Mol Genet. 2014;23(24):6458-69.
22. Hathout Y, Brody E, Clemens PR, Cripe L, DeLisle RK, Furlong P, et al. Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy. Proc Natl Acad Sci U S A. 2015.
23. Nadarajah VD, van Putten M, Chaouch A, Garrood P, Straub V, Lochmuller H, et al. Serum matrix metalloproteinase-9 (MMP-9) as a biomarker for monitoring disease progression in Duchenne muscular dystrophy (DMD). Neuromuscul Disord. 2011;21(8):569-78.
24. Hirata A, Masuda S, Tamura T, Kai K, Ojima K, Fukase A, et al. Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: a role for osteopontin. Am J Pathol. 2003;163(1):203-15.
25. Anaya-Segura MA, Garcia-Martinez FA, Montes-Almanza LA, Diaz BG, Avila-Ramirez G, Alvarez-Maya I, et al. Non-Invasive Biomarkers for Duchenne Muscular Dystrophy and Carrier Detection. Molecules. 2015;20(6):11154-72.
26. Rouillon J, Zocevic A, Leger T, Garcia C, Camadro JM, Udd B, et al. Proteomics profiling of urine reveals specific titin fragments as biomarkers of Duchenne muscular dystrophy. Neuromuscul Disord. 2014;24(7):563-73.
27. Zaharieva IT, Calissano M, Scoto M, Preston M, Cirak S, Feng L, et al. Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular Dystrophy. PLoS One. 2013;8(11):e80263.
28. Matsuzaka Y, Kishi S, Aoki Y, Komaki H, Oya Y, Takeda S, et al. Three novel serum biomarkers, miR-1, miR-133a, and miR-206 for Limb-girdle muscular dystrophy, Facioscapulohumeral muscular dystrophy, and Becker muscular dystrophy. Environmental health and preventive medicine. 2014;19(6):452-8.
29. Saito F, Saito-Arai Y, Nakamura A, Shimizu T, Matsumura K. Processing and secretion of the N-terminal domain of alpha-dystroglycan in cell culture media. FEBS Lett. 2008;582(3):439-44.
30. Singh J, Itahana Y, Knight-Krajewski S, Kanagawa M, Campbell KP, Bissell MJ, et al. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. Cancer Res. 2004;64(17):6152-9.
31. Yoon JH, Xu R, Martin P. A Method to Produce and Purify Full-Length Recombinant Alpha Dystroglycan: Analysis of N- and O-Linked Monosaccharide Composition in CHO Cells with or without LARGE Overexpression. PLoS currents. 2013;5.
32. Hesse C, Johansson I, Mattsson N, Bremell D, Andreasson U, Halim A, et al. The N-terminal domain of alpha-dystroglycan, released as a 38 kDa protein, is increased in cerebrospinal fluid in patients with Lyme neuroborreliosis. Biochem Biophys Res Commun. 2011;412(3):494-9.

33. Saito F, Saito-Arai Y, Nakamura-Okuma A, Ikeda M, Hagiwara H, Masaki T, et al. Secretion of N-terminal domain of alpha-dystroglycan in cerebrospinal fluid. Biochem Biophys Res Commun. 2011;411(2):365-9.
34. Bozzi M, Morlacchi S, Bigotti MG, Sciandra F, Brancaccio A. Functional diversity of dystroglycan. Matrix Biol. 2009;28(4):179-87.
35. Heng S, Vollenhoven B, Rombauts LJ, Nie G. A High-Throughput Assay for the Detection of alpha-Dystroglycan N-Terminus in Human Uterine Fluid to Determine Uterine Receptivity. J Biomol Screen. 2016;21(4):408-13.
36. Needham M, Mastaglia FL. Sporadic inclusion body myositis: a continuing puzzle. Neuromuscul Disord. 2008;18(1):6-16.
37. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science. 1989;244(4912):1578-80.
38. Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, Davies KE. Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature. 1996;384(6607):349-53.
39. Rafael JA, Tinsley JM, Potter AC, Deconinck AE, Davies KE. Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. Nat Genet. 1998;19(1):79-82.
40. Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, et al. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell. 1997;90(4):717-27.
41. Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR. Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell. 1997;90(4):729-38.
42. Ohlendieck K, Ervasti JM, Matsumura K, Kahl SD, Leveille CJ, Campbell KP. Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Neuron. 1991;7(3):499-508.
43. Matsumura K, Ervasti JM, Ohlendieck K, Kahl SD, Campbell KP. Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature. 1992;360(6404):588-91.
44. van Deutekom JC, Janson AA, Ginjaar IB, Frankhuizen WS, Aartsma-Rus A, Bremmer-Bout M, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med. 2007;357(26):2677-86.
45. Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhart PF, Heuvelmans N, et al. Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med. 2011;364(16):1513-22.
46. Mendell JR, Rodino-Klapac LR, Sahenk Z, Roush K, Bird L, Lowes LP, et al. Eteplirsen for the treatment of Duchenne muscular dystrophy. Ann Neurol. 2013;74(5):637-47.
47. Cirak S, Feng L, Anthony K, Arechavala-Gomeza V, Torelli S, Sewry C, et al. Restoration of the dystrophin-associated glycoprotein complex after exon skipping therapy in Duchenne muscular dystrophy. Mol Ther. 2012;20(2):462-7.
48. Finkel RS, Flanigan KM, Wong B, Bonnemann C, Sampson J, Sweeney HL, et al. Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy. PLoS One. 2013;8(12):e81302.

49. Wilton SD, Fletcher S, Flanigan KM. Dystrophin as a therapeutic biomarker: are we ignoring data from the past? Neuromuscul Disord. 2014;24(6):463-6.
50. Rezniczek GA, Konieczny P, Nikolic B, Reipert S, Schneller D, Abrahamsberg C, et al. Plectin 1f scaffolding at the sarcolemma of dystrophic (mdx) muscle fibers through multiple interactions with beta-dystroglycan. J Cell Biol. 2007;176(7):965-77.
51. McMillan JR, Akiyama M, Rouan F, Mellerio JE, Lane EB, Leigh IM, et al. Plectin defects in epidermolysis bullosa simplex with muscular dystrophy. Muscle Nerve. 2007;35(1):24-35.
52. Yoon JH, Chandrasekharan K, Xu R, Glass M, Singhal N, Martin PT. The synaptic CT carbohydrate modulates binding and expression of extracellular matrix proteins in skeletal muscle: Partial dependence on utrophin. Mol Cell Neurosci. 2009;41(4):448-63.
53. Chandrasekharan K, Yoon JH, Xu Y, deVries S, Camboni M, Janssen PM, et al. A human-specific deletion in mouse Cmah increases disease severity in the mdx model of Duchenne muscular dystrophy. Sci Transl Med. 2010;2(42):42ra54.
54. Flanigan KM, Dunn DM, von Niederhausern A, Soltanzadeh P, Gappmaier E, Howard MT, et al. Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. Hum Mutat. 2009;30(12):1657-66.
55. Chandraskeharan K, Martin PT. Embryonic overexpression of Galgt2 inhibits skeletal muscle growth via activation of myostatin signaling. Muscle Nerve. 2009;39(1):25-41.

FIGURE LEGENDS

Figure 1. Standard curves of 2A3 and 3B4 antibody binding to αDG-N protein using different ELISA assay methods. A) Different amounts of purified recombinant αDG-N protein were immobilized on an ELISA plate and binding of αDG-N-specific antibody 2A3 was assayed using a serum-immobilized ELISA. B) 2A3 was immobilized on an ELISA plate, and different amounts of unlabeled, recombinant αDG-N protein were premixed with a standard concentration of biotinylated αDG-N protein using a competition ELISA. C) 2A3 was immobilized on an ELISA plate, and different amounts of purified recombinant native αDG-N protein (aa30-312) or a fragment of αDG-N made in E. coli (αDG -GST, aa31-141) were added and then detected using a sandwich ELISA with a second αDG-N antibody, 3B4. D) Different amounts of purified recombinant αDG-N protein were immobilized and binding of 2A3 was assayed using a serum-immobilized ELISA. For the 5ng concentration of αDG-N, the sample was first incubated with or without 1ug/mL 3B4 prior to 2A3 addition. E) Different amounts of purified recombinant αDG-N protein were immobilized and binding of 3B4 was assayed using a serum-immobilized ELISA. For the 5ng concentration of αDG-N, the plate was first incubated with or without 1ug/mL 2A3 prior to 3B4 addition. For all curves, values are the average of duplicate readings. Points without error bars reflect error smaller than the size of the data point. Errors are SEM.

Figure 2. Total αDG-N ELISA signal and calculated serum concentration are decreased in serum from patients with DMD compared to normal. A) Otherwise normal adult (*n*=38), Duchenne Muscular Dystrophy (DMD, *n*=9), Becker Muscular Dystrophy (BMD, *n*=11), and Inclusion Body Myositis (IBM, *n*=8) patient serum samples were assayed for levels of αDG-N

using the serum-immobilized ELISA. Relative serum OD450 signal levels are expressed as fold change from the average of normal samples on the same plate. Each datum is the average of 3-7 independent measures per sample. B) Serum ELISA signals in A were used to calculate serum αDG-N concentration using αDG-N standard curves. * p <0.05, ** p <0.01, **** p <0.0001

Figure 3. Western blot analysis of αDG-N expression in normal human and DMD serum. αDG-N purified from cell lysate of transfected HEK293 cells, as well as 4 different DMD and 4 different otherwise normal age-matched human serum samples, were separated by SDS-PAGE and immunoblotted with the 2A3 (αDG-N) antibody or an antibody to fetuin. (B) αDG-N purified from transfected HEK293 lysate and supernatant, along with human and DMD serum, were compared with or without deglycosylation to remove both N- and O-linked glycans. For both A and B, anti-fetuin blots were done as a control for serum protein loading and transfer.

Figure 4. Serum αDG-N signal is decreased in mdx *Utrn*$^{-/-}$ mice relative to wild type or *mdx* mice. Serum samples from wild type (WT, n=11), mdx (n=10), and mdx *Utrn*$^{-/-}$ (n=4) mice were assayed for expression of αDG-N using the serum-immobilized ELISA. (A) Expression of relative ELISA signal is reported as fold change from the average of all WT samples assayed on the same plate. (B) Calculated serum concentrations of αDG-N. Each datum is the average of 2-4 independent measures per sample. ** p <0.01, *** p <0.001, **** p <0.0001

Figure 5. Schematic of αDG-N processing and potential changes in DMD. Dystroglycan is normally present within the dystrophin-associated glycoprotein complex, which includes dystrophin as a link between F-actin, syntrophins, dystroglycan (DG), which contains both an α and a β chain, sarcoglycans, and other proteins. In the lumen of the Golgi, α dystroglycan is cleaved by furin proteases to liberate the N-terminal domain of the protein, αDG-N, which goes on to be secreted into the serum from muscle and other tissues. In DMD, dystrophin protein is not expressed, and serum αDG-N levels are reduced in the serum using a serum-immobilized ELISA assay. This may reflect reduced intracellular DG expression or stability in DMD muscle, reduced αDG-N stability in DMD muscle or serum, reduced αDG-N secretion from DMD muscle, or increased αDG-N scavenging in DMD serum. Because αDG-N is immobilized for the ELISA measure, reduced αDG-N in DMD serum may also reflect increased masking of αDG-N by other DMD proteins.

Supplemental Figure 1. Signals from serum αDG-N ELISA are independent of age in otherwise normal, BMD and DMD patients, and are independent of gender for normal patients. Serum samples from otherwise normal (*n*=38)(A), BMD (*n*=11) (B) or DMD (*n*=9)(C) patients were assayed for expression of αDG-N via serum-immobilized ELISA. (D) Signals from αDG-N serum-immobilized ELISA assays of otherwise normal patients were compared by gender. Each datum is the average of 3-7 independent measures per sample.

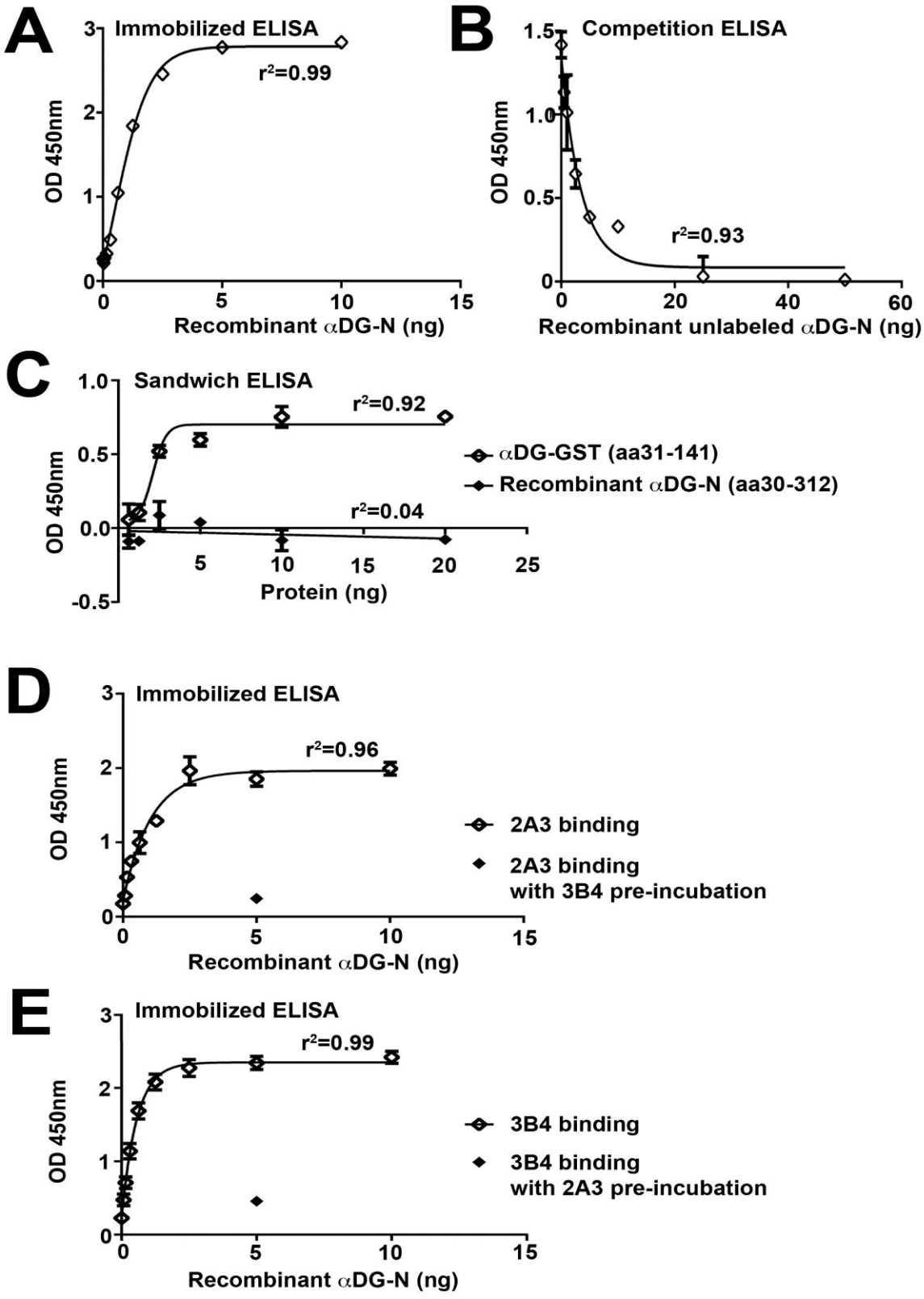

A
Immobilized ELISA
r²=0.99
OD 450nm
Recombinant αDG-N (ng)
B
Competition ELISA
r²=0.93
OD 450nm
Recombinant unlabeled αDG-N (ng)
C
Sandwich ELISA
r²=0.92
r²=0.04
αDG-GST (aa31-141)
Recombinant αDG-N (aa30-312)
OD 450nm
Protein (ng)
D
Immobilized ELISA
r²=0.96
2A3 binding
2A3 binding
with 3B4 pre-incubation
OD 450nm
Recombinant αDG-N (ng)
E
Immobilized ELISA
r²=0.99
3B4 binding
3B4 binding
with 2A3 pre-incubation
OD 450nm
Recombinant αDG-N (ng)

Figure 1

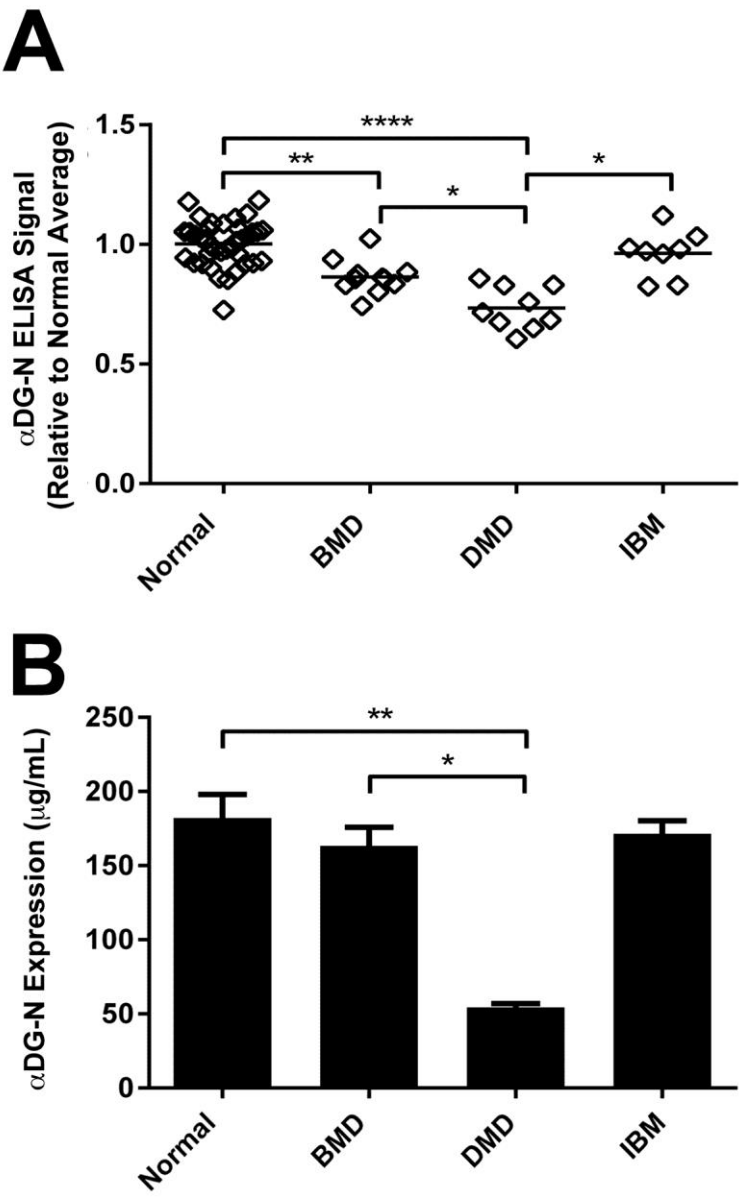

A
αDG-N ELISA Signal
(Relative to Normal Average)
1.5
1.0
0.5
0.0
****
**
*
*
Normal
BMD
DMD
IBM
B
αDG-N Expression (μg/mL)
250
200
150
100
50
0
**
*
Normal
BMD
DMD
IBM

Figure 2

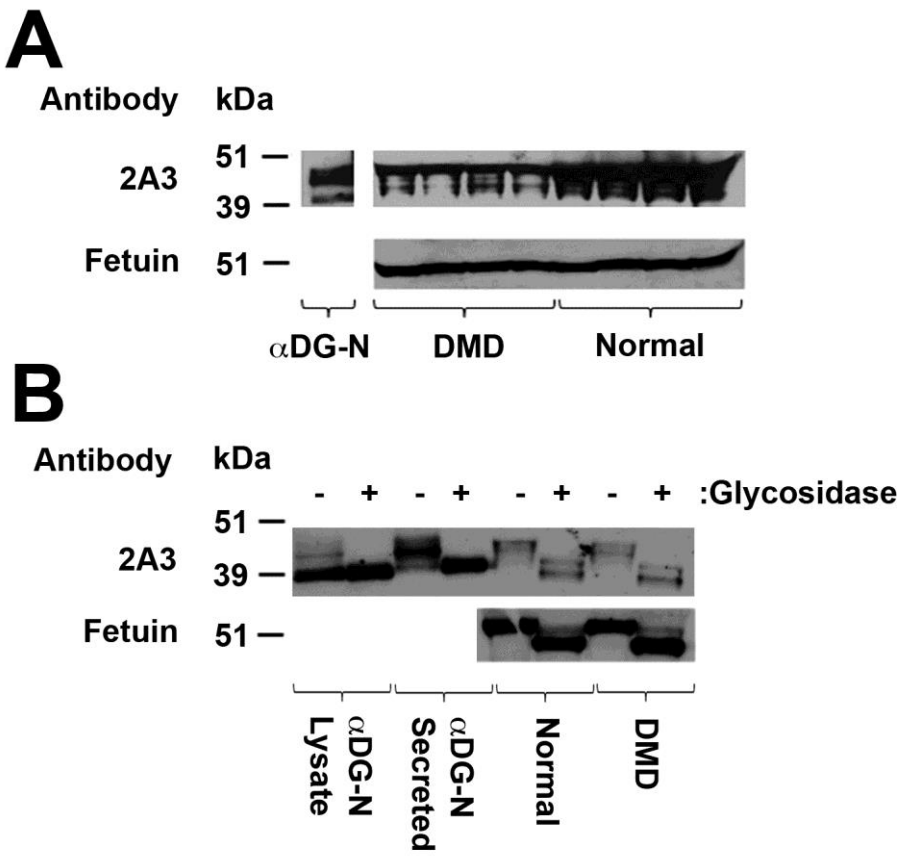

A
Antibody
kDa
2A3
51
39
Fetuin
51
αDG-N
DMD
Normal
B
Antibody
kDa
- + - + - + - + :Glycosidase
51
2A3
39
Fetuin
51
Lysate
αDG-N
Secreted
αDG-N
Normal
DMD

Figure 3

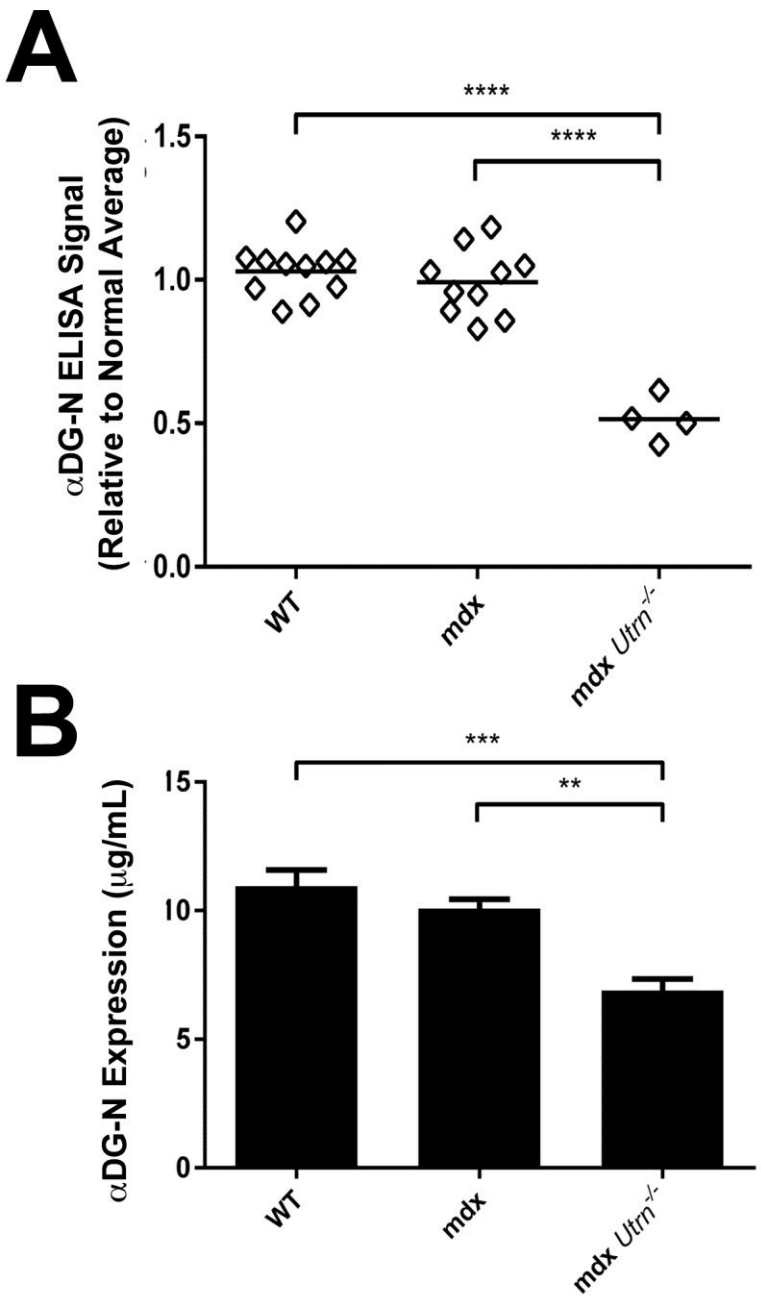

A
****
****
αDG-N ELISA Signal
(Relative to Normal Average)
1.5
1.0
0.5
0.0
WT
mdx
mdx Utrn-/-
B
***
**
αDG-N Expression (μg/mL)
15
10
5
0
WT
mdx
mdx Utrn-/-

Figure 4

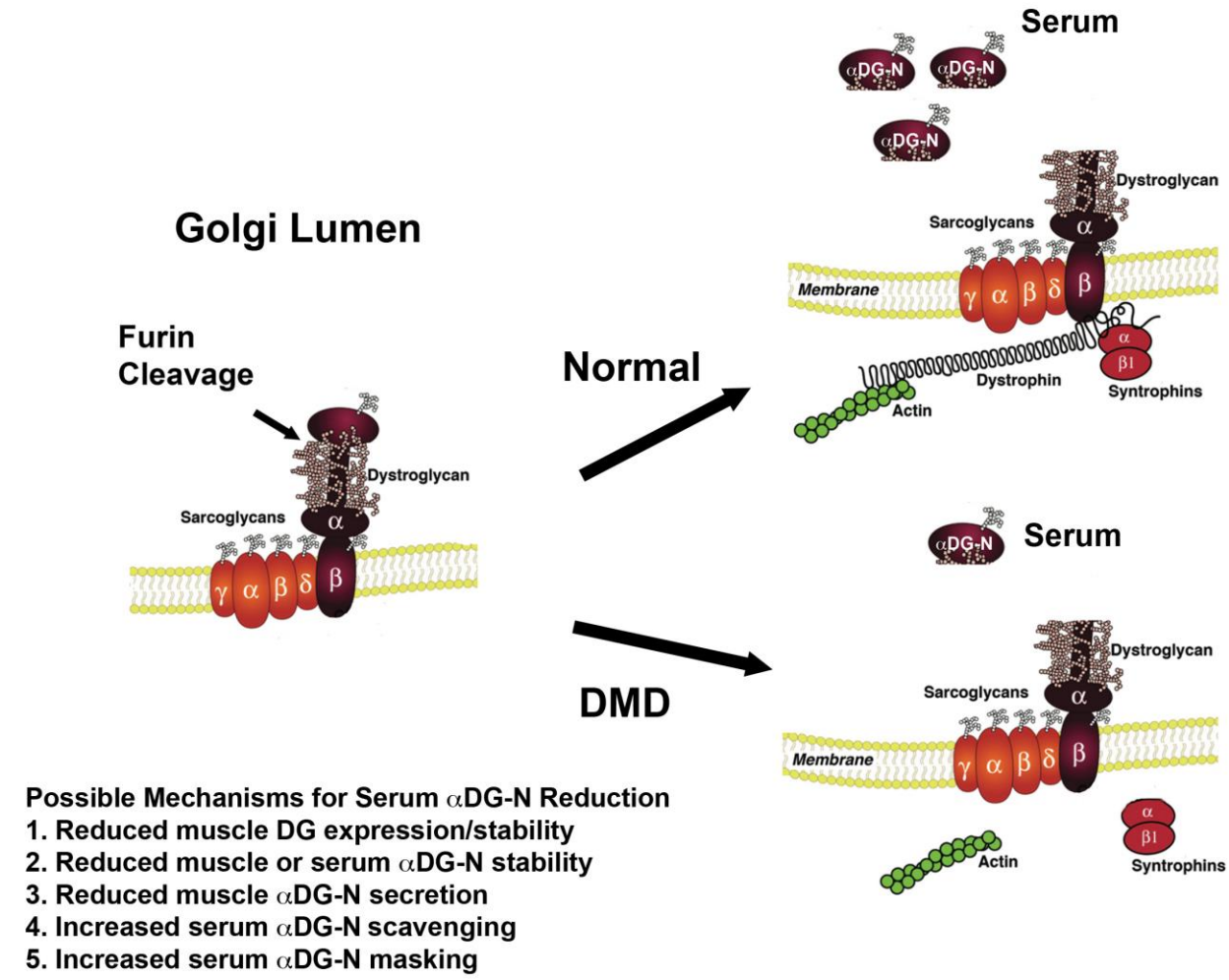
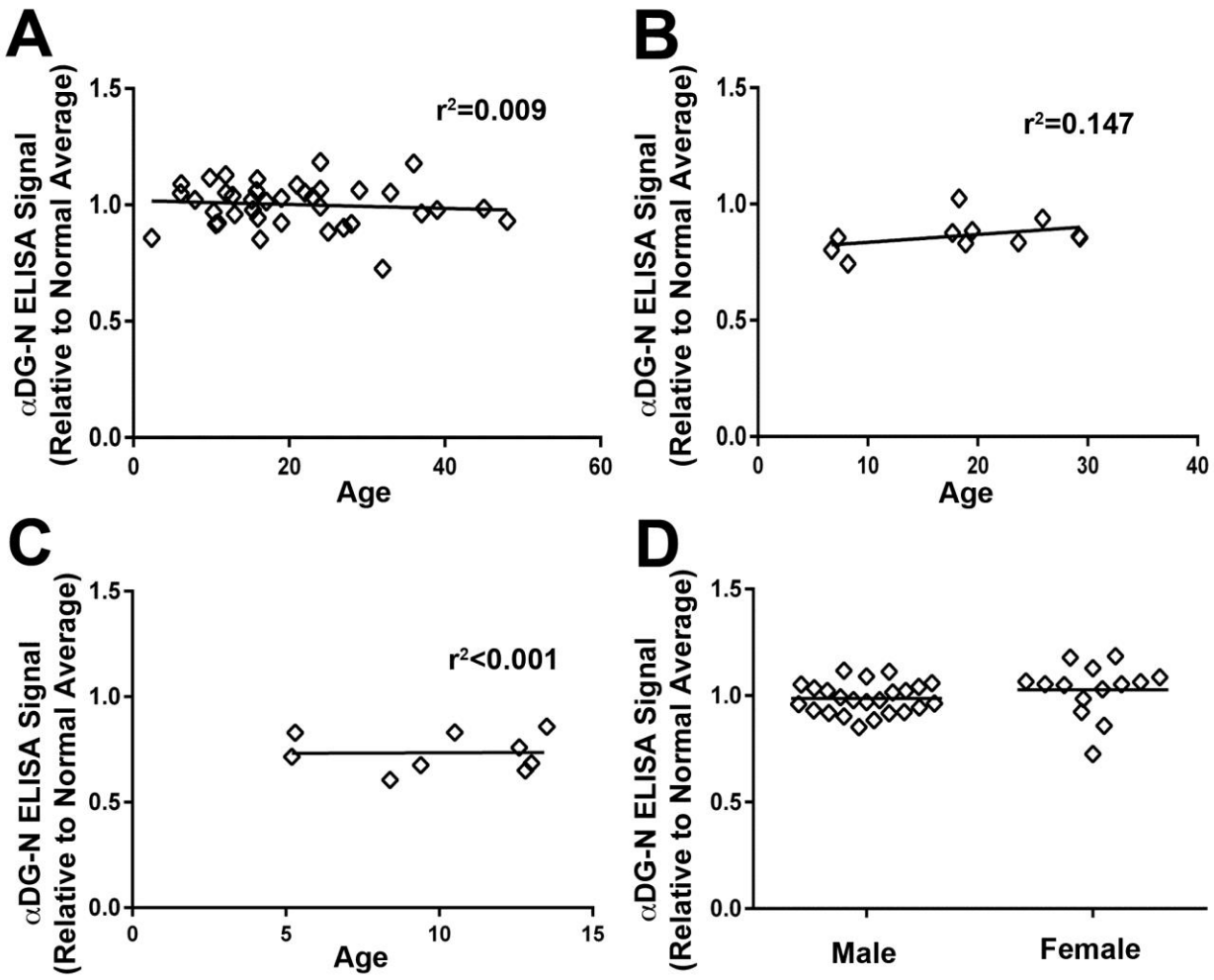

Golgi Lumen
Furin
Cleavage
Dystroglycan
Sarcoglycans
Normal
Serum
αDG-N
αDG-N
αDG-N
Dystroglycan
Sarcoglycans
Membrane
Dystrophin
Actin
Syntrophins
DMD
Serum
αDG-N
Dystroglycan
Sarcoglycans
Membrane
Actin
Syntrophins
Possible Mechanisms for Serum αDG-N Reduction
1. Reduced muscle DG expression/stability
2. Reduced muscle or serum αDG-N stability
3. Reduced muscle αDG-N secretion
4. Increased serum αDG-N scavenging
5. Increased serum αDG-N masking

Figure 5



Supplemental Figure 1.

Group	Patient	Gender	Years of Age	Ambulatory or Non-Ambulatory	On Steroids 3 Months Prior to Collection?	Mutation	Exon	Reading Frame
DMD	B010	M	5.2	Ambulatory	Yes	Nonsense	66	n/a
	B022	M	5.3	Ambulatory	Yes	Deletion	48-50	Out of Frame
	B014	M	8.4	Ambulatory	Yes	Nonsense	10	n/a
	B012	M	9.4	Ambulatory	Yes	Duplication	19	Out of Frame
	B007	M	10.5	Ambulatory	Yes	Deletion	3-7	Out of Frame
	B027	M	12.6	Non-Ambulatory	Yes	Duplication	21	Out of Frame
	B002	M	12.8	Ambulatory	Yes	Deletion	51	Out of Frame
	B023	M	13.0	Non-Ambulatory	Yes	Deletion	12-13	Out of Frame
	B008	M	13.5	Non-Ambulatory	No	Deletion	45-46	In Frame
BMD	B041	M	6.7	Ambulatory	No	Deletion	45-55	In Frame
	B036	M	7.3	Ambulatory	Yes	Deletion	3-5	In Frame
	B040	M	8.2	Ambulatory	Yes	Deletion	45-55	In Frame
	B038	M	17.7	Ambulatory	No	Deletion	45-47	In Frame
	B039	M	18.3	Ambulatory	No	Nonsense	28	n/a
	B013	M	18.9	Non-Ambulatory	No	Deletion	5	In Frame
	B016	M	19.5	Ambulatory	No	Deletion	45-47	In Frame
	B037	M	23.7	Ambulatory	No	Deletion	45-48	In Frame
	B005	M	25.9	Non-Ambulatory	No	Nonsense	29	n/a
	B006	M	29.2	Non-Ambulatory	No	Nonsense	29	n/a
	B004	M	29.3	Ambulatory	No	Duplication	2-7	In Frame
IBM	B015	M	52.4	Ambulatory	No			
	B020	M	58.9	Ambulatory	No			
	B017	M	64.0	Ambulatory	No			
	B011	M	65.5	Ambulatory	No			
	B021	M	67.1	Ambulatory	No			
	B018	M	71.9	Ambulatory	No			
	B019	M	79.0	Ambulatory	No			
	B024	M	80.1	Ambulatory	No			
Normal	HC14	F	2.4					
	HC13	F	6.1					
	HC05	M	6.2					
	HC10	M	7.9					
	HC11	M	9.8					
	HC04	M	10.3					
	HC12	M	10.6					
	HC03	M	10.9					
	HC15	F	11.9					
	HC06	M	11.9					
	HC07	M	12.8					
	HC19	M	13.0					
	HC02	M	15.2					
	HC18	M	15.4					
	HC08	M	15.8					
	HC09	M	15.9					
	HC16	M	16.0					
	HC01	M	16.3					
	HC17	M	17.1					
	N0033	F	19					
	N9049	F	19					
	N9047	F	21					
	N9051	F	22					
	N0037	M	23					
	N0031	F	24					
	N0035	F	24					
	N0039	M	24					
	N9041	M	25					
	N9045	M	27					
	N9044	M	28					
	N9048	F	29					
	N0034	F	32					
	N9050	F	33					
	N0032	F	36					
	N9043	M	37					
	N9042	M	39					
	N9052	F	45					
	N9046	M	48					

Supplemental Table 1